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ORIGINAL PAPER

# The role of chromosomal rearrangements in the evolution of *Silene latifolia* sex chromosomes

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**Abstract** *Silene latifolia* is a model plant for studies of the early steps of sex chromosome evolution. In comparison to mammalian sex chromosomes that evolved 300 mya, sex chromosomes of *S. latifolia* appeared approximately 20 mya. Here, we combine results from physical mapping of sex-linked genes using polymerase chain reaction on microdissected arms of the *S. latifolia* X chromosome, and fluorescence in situ hybridization analysis of a new cytogenetic marker, *Silene* tandem repeat accumulated on the Y chromosome. The data are interpreted in the light of current genetic linkage maps of the X chromosome and a physical map of the Y chromosome. Our results identify the position of the centromere relative to the mapped genes on the X chromosome. We suggest that the evolution of the *S. latifolia* Y chromosome has been accompanied by at least one paracentric and one pericentric inversion. These results indicate that large chromosomal rearrangements have played an important role in Y chromosome evolution in *S. latifolia* and that chromosomal rearrangements are an integral part of sex chromosome evolution.

**Keywords** Chromosomal rearrangements · Sex chromosomes · Microdissection · FISH

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## Introduction

Sex chromosomes have been proposed to originate from an ordinary pair of autosomes that have stopped recombination (step by step) and gradually diverged from each other (Ohno 1967). The evolution of sex chromosomes is accompanied by the degeneration of genes on the Y chromosome and the accumulation of various classes of repetitive DNA sequences in non-recombining regions (Charlesworth 1991). Such non-recombining regions are thought to have formed as a result of the chromosomal rearrangements in evolving sex chromosomes as they contribute to the suppression of recombination.

*Silene latifolia* has become a model organism to study early events during the evolution of sex chromosomes. Heteromorphic sex chromosomes of *S. latifolia* were independently identified by Blackburn (1923) and Winge (1923). In comparison to mammalian sex chromosomes that evolved 300 mya, sex chromosomes of *S. latifolia* appeared approximately 20 mya (Atanassov et al. 2001). The X chromosome is submetacentric with cytologically distinguishable p and q arms, whereas the Y chromosome is metacentric and about 1.4× the size of the X chromosome (Vyskot and Hobza 2004).

In contrast to the highly degenerated human Y chromosome, *S. latifolia* possesses a largely euchromatic Y chromosome, with the exception of centromeric and subtelomeric regions. Based on cytogenetic studies, the pseudoautosomal region (PAR) is located at the short subtelomeric region of the p arm on the X chromosome, and the q arm of the Y chromosome (Lengerova et al. 2003). Although the *S. latifolia* Y chromosome is largely non-heterochromatic, it has accumulated specific repetitive DNA elements on both of its arms (Hobza et al. 2004, 2006a; Kejnovsky et al. 2006) and contains Y-chromosome-specific low copy sequences (Hobza et al. 2006b).

Several genes with homologous X- and Y-linked copies and a pseudoautosomal marker were recently identified and characterized in *S. latifolia*, including *SlX1/Y1* (Delichere et al. 1999), *SlX4/Y4* (Atanassov et al. 2001), *DD44X/Y* (Moore et al. 2003), *SlX3/Y3* (Nicolas et al. 2005), *SlssX/Y* (Filatov 2005b), and OPA-09 (Di Stilio et al. 1998). A genetic linkage map of the X chromosome has been recently constructed by Nicolas et al. (2005) for *S. latifolia* and two closely related dioecious species, *S. dioica* and *S. diclinis*. Filatov (2005a) independently compared genetic maps of the *S. latifolia* X-chromosome and a potentially homologous chromosome in the gynodioecious species *S. vulgaris*. The results support Ohno's hypothesis (Ohno 1967) that sex chromosomes evolved from a single pair of autosomes. The comparison of the homologous X- and Y-linked copies of mapped genes further revealed that different gene regions might have ceased recombination independently during evolution, which has led to the formation of evolutionary strata on these chromosomes (Nicolas et al. 2005; Bergero et al. 2007).

Unfortunately, the potential for comparative genetic mapping between the X and Y chromosomes is restricted to short recombining region(s). Lebel-Hardenack et al. (2002) overcame this problem by generating a collection of Y chromosome deletion mutants. The use of mutants for AFLP mapping of male co-segregating markers enabled them to construct a deletion map of the Y chromosome. This map was later extended by Moore et al. (2003) who added previously characterized Y copies of sex-linked genes (*SlY1*, *SlY4*, *DD44Y*). Recently, a comparison of the X and Y chromosome maps led to a conclusion that a large inversion occurred during the evolution of the *S. latifolia* Y chromosome (Zluvova et al. 2005). However, the results of these mapping studies did not reveal the number, extent and character, i.e., para- and/or pericentric inversion(s), of the rearrangements. Here, we further characterize gene positions on the *S. latifolia* sex chromosomes using polymerase chain reaction (PCR) on microdissected arms of X chromosomes. In combination with fluorescence in situ hybridization (FISH) mapping of a newly characterized cytogenetic marker, we show that at least two large chromosomal rearrangements have occurred during the evolution of these plants sex chromosomes.

## Materials and methods

### Plant material and isolation of metaphase chromosomes

*Silene latifolia* Garcke plant material was obtained from a seed collection of the Institute of Biophysics, Brno. Sterilized seeds were cultured for 2 days in distilled water and then synchronized with aphidicoline (30 mmol/l for 12 h) and oryzalin (15 µmol/l for 4 h). Root tips from germinating seedlings were cut off and enzymatically transformed into protoplasts. The protoplasts were briefly fixed in the mixture of ethanol:acetic acid (3:1) to avoid further DNA damage. The mitotic protoplast suspension was dropped on polyethylene naphthalate membrane (for laser microdissection, stained with Giemsa) or on microscope slides (for FISH experiments), where naked chromosomes were released (Hobza and Vyskot 2007).

### Laser microdissection and I-PEP-PCR amplification

The microdissection experiments were performed according to Hobza et al. (2004) with minimum incubation of chromosomes in the fixative (5 min) to avoid impairment of DNA. The PALM MicroLaser system (P. A. L. M. MnbH, Bernried, Germany) was applied to isolate chromosome parts of the X. The system consists of a 337 nm nitrogen laser coupled to the light path of an inverted microscope. A 1.5–11.7 mJ energy pulse was used for dissection and a 2 mJ pulse for catapulting. Improved primer-extension-preamplification polymerase chain reaction (I-PEP-PCR) technology was used to amplify the template (Matsunaga et al. 1999). Briefly, we performed 50 cycles with a denaturation step at 94°C for 1 min, an annealing step at 30°C for 2 min with a transit step for 3 min to 55°C, an extension step at 55°C for 4 min with a second extension step at 68°C for 30 s.

### Polymerase chain reaction detection of X-linked gene sequences

Improved primer-extension-preamplification-PCR amplified p and q arms of the X chromosome (15 arms/reaction) were purified using Qiagen PCR purification kit and used as a template for subsequent PCR. The reactions were performed in a volume of 50 µl, and the final concentration of reagents was: 0.2 mM dNTP, 0.2 µM primers (see bellow), 1 × buffer containing 1.5 mM $MgCl_2$, and 0.6 U of *Taq* polymerase (Promega). Initial denaturation was followed by 25 cycles of 50 s at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension for 7 min. The PTC-200 thermal cycler (MJ Research) was used. Following primers were used to amplify X-linked genes:

*Sl1* gene:

57S35 (5′-TGG ACT TCC ACT GGA ATT CGA T-3′)
57vAS12 (5′-AGG GTG CCA TTT CAT TAC CCT C-3′)

*Sl3* gene:

Sl3ROM-f (5′-TGA TGG CTT TGT GGA TTT CA-3′)
Sl3ROM-r (5′-AAC TCA TGC TTG CCG ATC TC-3′)

*Sl4* gene:

95vS11 (5′-GTG GCC TGG GCG TCT ATG TG-3′)
95vAS3 (5′-AAT TAC CGA AGA CAG TAA AGC GTC-3′)

*DD44* gene:

DD44F1 (5′-GTG TTC GAC ATG TCC ATC AGA ACC-3′)
DD44R1 (5′-CCA TCA CTT CTT ATT TTA TGC AGG-3′)

Pseudoautosomal region marker:

OPASF1 (5′-GCA ATT CAC CAT CCT CTG CT-3′)
OPASHR1 (5′-ATG GTC TTT GGG CCC TTA TC-3′)

### Isolation of the STAR-Y marker

A plasmid genomic library was constructed to screen the highly abundant DNA elements in *S. latifolia* genome. First, male genomic DNA was sonicated and DNA fragments of average size 800 bp gels were extracted. Purified DNA was incubated with Exonuclease III and subsequently ligated with plasmid vector (*Sma*I cloning site of pBluescript SK-). Transformed competent cells (JM109) were robotically picked and transferred onto microtiter plates. Finally, bacteria were gridded on a nylon membrane (10,000 clones) and immobilized by alkaline treatment for subsequent hybridization. Membranes were hybridized (AlkPhos Direct hybridization kit, Amersham Pharmacia Biotech) with *S. latifolia* male genomic DNA and the most strongly hybridized clones were sequenced. Sequence analysis by JDotter (Brodie et al. 2004; http://www.athena.bioc.uvic.ca/pbr/jdotter/) revealed the presence of a tandem repeat STAR-C (Silene tandem repeat accumulated on centromeres) with a monomer length of 43 bp and its deleted variant STAR-Y (Silene tandem repeat accumulated on the Y chromosome) in 23 strongly hybridizing clones (see the sequences in Supplement No. 1).

### Fluorescence in situ hybridization on metaphase chromosomes

Polymerase chain reaction products were purified using Nucleotide Removal Kit (Qiagen) and labeled by nick translation using Nick Translation Mix (Roche) with Fluorolink Cy3-dUTP (Amersham Pharmacia Biotech). Slides were preheated at 60°C for 30 min, treated with 100 µg/ml RNase A (Sigma) in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 1 h at 37°C, washed three times for 5 min in 2 × SSC, treated with 5 mg/ml pepsin (Sigma) in 0.01 N HCl for 12 min at 37°C, washed as before, postfixed in 3.7% formaldehyde (Merck) in 1 × PBS for 10 min, washed again and dehydrated in increasing ethanol series (70%, 70%, 96% ethanol, 5 min each). The hybridization mixture contained 50% formamide (v/v, SigmaULTRA), 10% dextran sulfate (w/v, Sigma), 2 × SSC, and 10–100 ng of probe. The mixture was predenaturated by incubation at 75°C for 10 min and immediately placed on ice. Typically, 20 µl of the mixture was applied per slide and covered with a plastic coverslip. Controlled denaturation and annealing was done on a thermal cycler with heated platform by step-by-step incubation of slides in the following manner (75°C for 5 min → 65°C for 2 min → 55°C for 2 min → 45°C for 2 min → 37°C for 2 min). Slides were then incubated overnight at 37°C in a moist chamber. Posthybridization washing was done at 76% stringency by following steps: 2 × SSC (42°C, twice for 5 min), 0.1 × SSC (42°C, twice for 5 min), 2 × SSC (42°C, twice for 5 min), 4 × SSC + 0.1% Tween 20 (RT, 7 min). Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml) in Vectashield (Vector). Images were captured using a charge-coupled device (CCD) camera and ISIS software (MetaSystems). The longitudinal distribution of signals along the chromosomes was studied using ImagePro software (Media Cybernetics).

## Results

We separated the p and q arms of the X chromosome of *Silene latifolia* using laser microdissection (Fig. 1). The separated arms were subsequently used as a template for I-PEP-PCR to enlarge the amounts of available DNA and to allow direct PCR amplification of target genes using gene specific primers. The results of these PCR experiments enabled us to map all target genes to either the p or q arms of the X chromosome. The *SlX1* gene and OPA marker were amplified solely on the p arm of the X chromosome while *SlX4*, *SlssX*, *DD44X*, and *SlX3* genes were mapped on its q arm (Fig. 2). These data allowed us to improve the current genetic map(s) and revealed the position of the analyzed genes with respect to the centromere.

A comparison of our results with those reported by others (Nicolas et al. 2005; Filatov 2005a; Zluvova et al. 2005) reveals the position of the centromere within the X chromosome in relation to the mapped genes. Nicolas et al. (2005) and Filatov et al. (2005a) employed genetic mapping to reveal the position of the studied genes on the X chromosome. Zluvova et al. (2005) mapped the position of several sex chromosome linked genes on the Y chromosome using PCR on DNA from Y chromosome deletion mutants. These mutant plants were previously generated by Lebel-Hardenack et al. (2002) by induction of sexual phenotype mutants using X-ray irradiation. The authors deduced positions of the genes on the p and q arm of the Y chromosome, localized

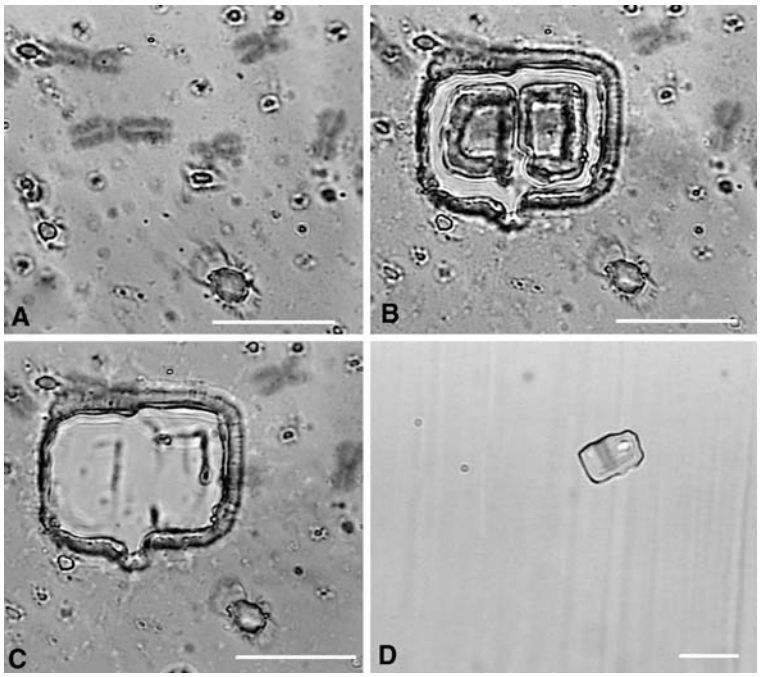


**Fig. 1** Laser microdissection of the p and q arms of the *S. latifolia* X chromosome. Metaphase protoplasts were dropped on a polyethylene naphthalate membrane and stained with Giemsa. A suitable X chromosome was localized under the inverted microscope (**a**). The membrane was cut around the selected region using a laser microbeam (**b**) and the chromosomal arm was catapulted by a single laser pulse (**c**) into the cap of a PCR tube (**d**). *Bars* represent 10 µm

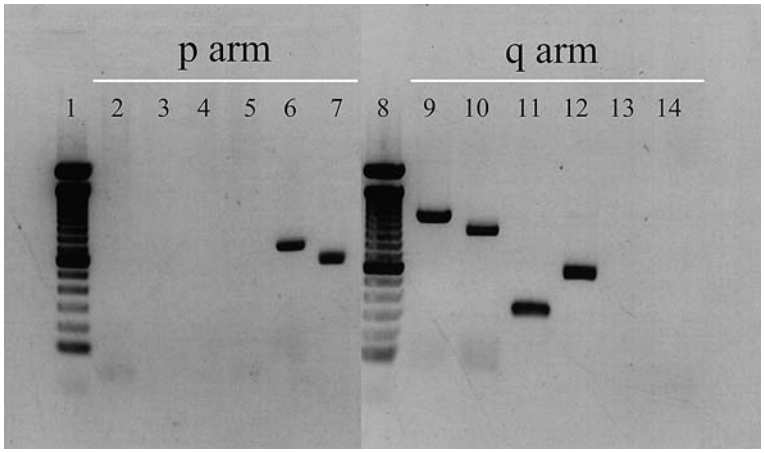


**Fig. 2** Localization of sex chromosome-linked genes by PCR on the p and q arms of the X chromosome. Microdissected chromosomal DNA was amplified using I-PEP-PCR followed by PCR with a gene specific primer pairs. The products of I-PEP-PCR reactions were diluted 100× and 1 µl was used as a template for PCR with gene-specific primers. The template DNA (either the p arm or q arms) is indicated in the figure (individual lines 1, 8—size marker (100 bp); 2, 9—*SlX4*; 3, 10—*SlssX*; 4, 11—*DD44X*; 5, 12—*SlX3*; 6, 13 pseudoautosomal sequence OPA; 7, 14—*SlX1*). PCR products were subjected to electrophoresis on 1% agarose gel and stained with SYBR green

the centromeric region with respect to the analyzed genes and described the order of genes on the Y chromosome. Their data suggest at least one inversion event occurred on the Y chromosome during evolution of sex chromosomes in *S. latifolia*. Based on these results we propose a model for the evolution of the *S. latifolia* Y chromosome that includes at least two inversions (Fig. 3). First, a paracentric inversion occurred on the p arm of the Y chromosome where originally all mapped genes except *SlY1* (located on the q arm) were localized. Subsequently, a second pericentric inversion relocated gene *SlY4* from the p arm of the Y chromosome to its q arm.

This model is supported by FISH mapping of a newly characterized marker, STAR-Y (*Silene* tandem repeat accumulated on the Y chromosome). We isolated this DNA sequence by screening a plasmid genomic library with male genomic DNA while searching for transposable elements and satellites. Several of the stronger hybridizing clones contained a satellite with a monomer length of 43 bp STAR-C, which was localized on all centromeres by FISH. In addition, we found its deleted version with a monomer length 29 bp (STAR-Y), which was strongly accumulated within specific regions of the Y chromosome—the central part of the q arm and the subtelomeric region of the p arm (Fig. 4).

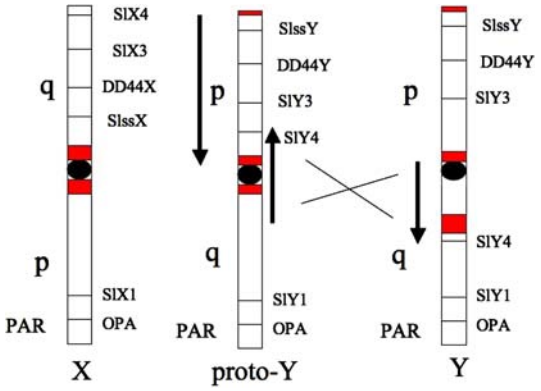


**Fig. 3** A model indicating the evolutionary steps that led to the formation of sex chromosomes in *S. latifolia*. *Arrows* indicate regions that underwent chromosomal inversions. *Red rectangles* indicate the position of the STAR-Y repetitive DNA. The order of genes on the X chromosome corresponds most likely to the original arrangement of genes on ancestral autosomes from which the sex chromosomes evolved (according to Nicolas et al. 2005, and this article). The proto-Y chromosome shows the arrangement of genes and the STAR-Y repeats on the evolving Y chromosome after the first paracentric inversion. The second pericentric inversion formed the current Y chromosome. The order of Y-genes comes from Zluvova et al. (2005). The position of the STAR-Y repeats within the q arm of the Y chromosome may be a result of not only the inversion event but also the expansion of other Y chromosome centromeric repeats (not to scale)

## Discussion

In this work we mapped genes onto the p or q arms of the X chromosome in *S. latifolia*. Based on these data combined with the new cytogenetic marker localized on the Y chromosome we suggest a new model of the Y chromosome evolution involving two large inversions, one paracentric and one pericentric.

Here, we propose that the hybridization pattern of a new cytogenetic marker STAR-Y is a consequence of the suggested inversions on the Y chromosome. First, the relocation of STAR-Y from the centromere to the subtelomere of the Y chromosome's p arm was probably caused by a large inversion that involved the entire chromosomal arm. This inversion led to the observed opposite orders of mapped genes on the X and Y chromosomes. Second, a pericentromeric inversion on the Y chromosome led to the transfer of *SlY4* from the p arm to the q arm and may have initiated the arrest of recombination in the q arm. The occurrence of STAR-Y on the q arm of the Y chromosome, away from the centromeric region, may be a consequence of the expansion of other repetitive DNA sequences upon the arrest of recombination. This expansion presumably moved STAR-Y to a more central part of the q chromosomal arm. STAR-Y itself has spread along the non-recombining part of the Y chromosome and it is one of the elements whose expansion may account for the large size of the Y chromosome (Hobza et al. 2006a).

Both FISH experiments with the newly identified repetitive sequence STAR-Y and physical mapping of X-linked genes using PCR on microdissected chromosomal arms, revealed that at least two large inversions have contributed to Y chromosome formation and thus to sex chromosome evolution in *S. latifolia*. The roles of both chromosome inversions (Zluvova et al. 2005) and translocations (Matsunaga et al. 2003) have been found in the evolutionary history of the Y chromosome in *S. latifolia*. Similarly, several inversions have occurred on the human Y chromosome in the course of its evolution where these inversions created different "evolutionary strata" on human X chromosome (Lahn and Page 1999). Our data thus support the view that chromosomal rearrangements are an integral part of sex chromosome evolution.

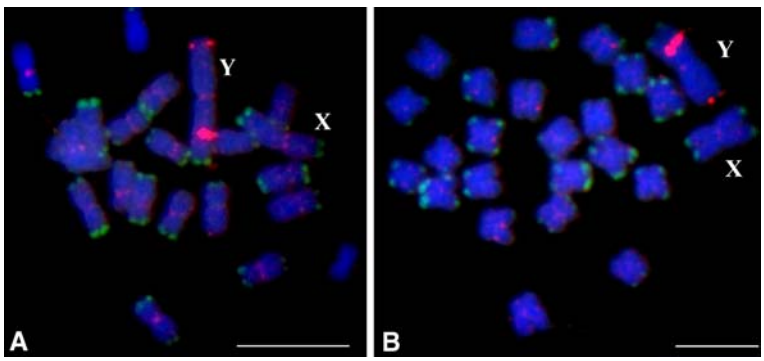


**Fig. 4** Chromosomal distribution of the STAR-Y sequence revealed by FISH experiment. Metaphase chromosomes of *S. latifolia* male were hybridized with the STAR-Y DNA probe. Chromosomes were counterstained with DAPI (*blue*); the probe was labeled with Cy3-conjugated nucleotides (*red*). *Green signal* presents the marker X43.1 probe distinguishing the q arm of the Y chromosome (Buzek et al. 1997). The X and Y chromosomes are indicated, *bars* indicate 10 μm

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